

QUALITY ASSURANCE PROJECT PLAN

NOOKSACK RIVER WATERSHED

MICROBIAL SOURCE TRACKING STUDY

Version 1.0

Water Resources Division
Natural Resources Department
Lummi Indian Business Council

Natural Resources
Whatcom County Public Works

EPA Region 10 Laboratory

December 2015

Nooksack River Watershed Microbial Source Tracking Study Quality Assurance Project Plan Approval (A1):




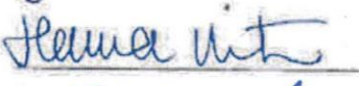



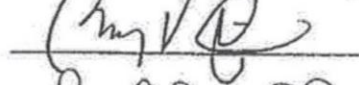
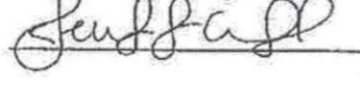
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1. DISTRIBUTION LIST (A3)

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Recipients identified on this distribution list will be sent updated versions of this document or copies of minor change letters for this document immediately after they are approved.

Recipients listed may also obtain the most current copy of the approved Quality Assurance Project Plan (QAPP) by contacting the Lummi Nation Water Resources Manager or Water Resources Specialist II or from the Lummi Natural Resources Department website through the following link: <http://lnnr.lummi-nsn.gov/LummiWebsite/Website.php?PageID=56>.

Final laboratory results will be distributed by Stephanie Bailey, to individuals marked with an asterisk in the distribution list above.

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2. PROJECT ORGANIZATION (A4)

The following individuals are responsible for the design and implementation of this project, and/or will be the primary data users and decision makers:

- **Leroy Deardorff**, Lummi Natural Resources Department Deputy Director, is responsible for ensuring that the Lummi Water Resources Division has the necessary resources and effectively fulfills the roles and responsibilities identified in this QAPP.
- **Jeremy Freimund P.H.**, Lummi Nation Water Resources Manager, is responsible for overseeing Lummi Natural Resources Department (LNR) Water Resources Division (LWRD) employees who will implement the Nooksack River Watershed Microbial Source Tracking (MST) Study and will serve as the Quality Assurance Manager for LWRD-collected samples.
- **Jamie Mattson**, Lummi Nation Water Resources Specialist II, is responsible for overseeing the work of the Water Resources Specialist I and serves as the Quality Assurance Officer for LWRD-collected samples.
- **Hanna Winter**, Lummi Nation Water Resources Specialist I, is responsible for implementing the Nooksack River Watershed MST Study for the LWRD, including preparation and maintenance of the official, approved Quality Assurance Project Plan (QAPP), data collection, and sample delivery to the Environmental Protection Agency (EPA) Region 10 Environmental Laboratory, and overseeing the Water Resources Technician III.
- **Lisa Cook**, Lummi Nation Water Resources Technician III, is responsible for assisting the Water Resources Specialist I with sample collection, tracking, and delivery, including performing quality assurance/quality control (QA/QC) activities.
- **Stephanie Bailey**, EPA Region 10 Environmental Laboratory Microbiologist, is responsible for the preparation of the laboratory-specific sections of this QAPP, analysis of MST samples, and preparation of the final lab report.
- **Bill Zachmann**, EPA Region 10 Office of Water and Watersheds Project Manager, will serve as the primary point of contact at EPA Region 10 for the Nooksack River Watershed MST Study.
- **Jennifer Crawford**, EPA Region 10 Regional Sample Control Coordinator, will coordinate sample analyses performed by the EPA Region 10 Laboratory and will provide unique assigned project codes, Region 10 sample numbers, and training on Region 10 sample collection, documentation and shipment requirements to LWRD and Whatcom County staff implementing the Nooksack River Watershed MST Study.
- **Donald M. Brown**, EPA Region 10 Regional Quality Assurance Manager, is responsible for reviewing and approving the QAPP and any subsequent revisions and amendments. Responsibilities for quality assurance (QA) review are also be delegated to R10 QA

Chemists. For this project, Jennifer Crawford will provide QA support and oversight for the project.

- **Gary Stoyoka**, Whatcom County Public Works Natural Resources Program Manager, is responsible for overseeing Whatcom County employees who will implement the Nooksack River Watershed MST Study, including the Whatcom County Senior Planner, and will serve as the Quality Assurance Officer for Whatcom County-collected samples.
- **Erika Douglas**, Whatcom County Public Works Natural Resources Senior Planner, is responsible for implementing the Nooksack River Watershed MST Study for Whatcom County, including overseeing sample collection, tracking, and delivery to the EPA Region 10 Laboratory.

3. PROBLEM DEFINITION/BACKGROUND

The Nooksack River watershed comprises the majority of the Water Resources Inventory Area 1 (WRIA 1) located in Whatcom County in Washington State (Figure 3.1). From its headwaters in the northwestern Cascade Mountains, the Nooksack River drains approximately 809 square miles, comprising most of western Whatcom County, including agricultural areas and the developed lowlands surrounding the towns of Deming, Everson, Lynden, and Ferndale. The Nooksack River enters the Lummi Indian Reservation at its eastern extent, which contains the majority of the river delta before it discharges into the marine waters of Bellingham Bay. The Nooksack River is also the primary source of freshwater into Portage Bay, which is located approximately 5 miles southwest of the Nooksack River delta (DOH 1997).

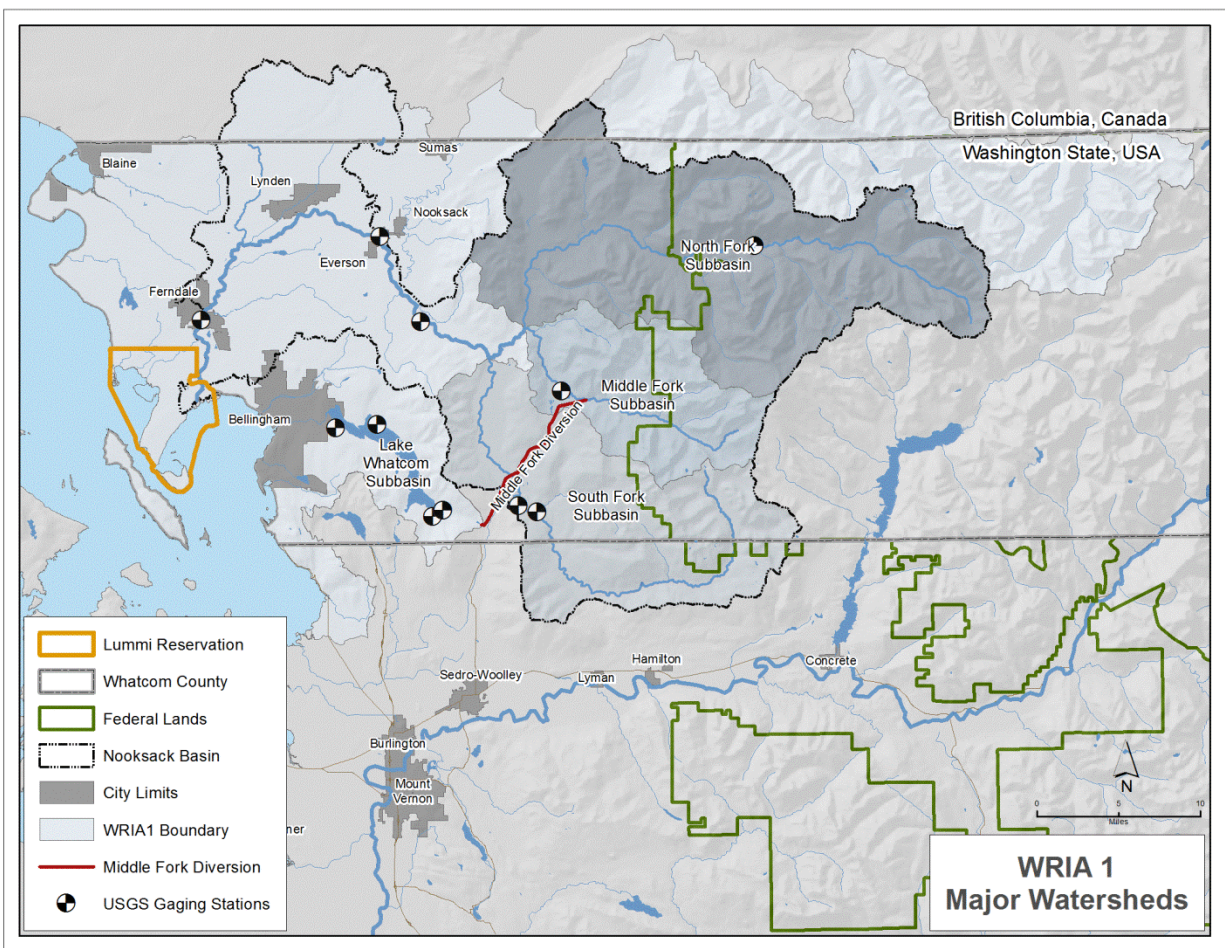


Figure 3.1 Regional Location of the Nooksack River Watershed and the Lummi Indian Reservation.

Portage Bay is located within the Lummi Indian Reservation boundaries and contains important shellfish beds harvested for commercial, cultural, and subsistence purposes by members of the

Lummi Nation. Fecal coliform contamination from the Nooksack River presently and historically has threatened Portage Bay shellfish growing areas and resulted in shellfish harvest closures.

In consultation with the Lummi Nation, pursuant to the Shellfish Consent Decree (Order Regarding Shellfish Sanitation, *United States v. Washington [Shellfish]*, Civil Number 9213, Subproceeding 89-3, Western District of Washington, 1994), the Washington State Department of Health (DOH) is responsible to the federal Food and Drug Administration (FDA) to ensure that the National Shellfish Sanitation Program (NSSP) standards for certification of shellfish growing waters are met on the Reservation. Fecal coliform concentrations and *in situ* water quality (temperature and salinity) have been monitored in Portage Bay by the Lummi Water Resources Division (LWRD) in partnership with the DOH since 1989. A total of 12 samples sites in Portage Bay are currently monitored. In addition, bacteria (fecal coliform, *E. coli*, and enterococcus) and *in situ* water quality parameters (temperature, pH, dissolved oxygen, specific conductivity, and salinity) have been monitored at several surface water quality sample sites on the Lummi Reservation, including the Nooksack River at Marine Drive Bridge, as part of the Lummi Nation Ambient Surface Water Quality Monitoring Program that was established in 1993. The program provides data regarding the water quality and bacteria levels of waters entering Portage Bay from the Nooksack River and from on-Reservation sources. There are currently 18 surface water quality samples sites in and draining into Portage Bay in this ambient monitoring program that are sampled on a regular basis.

The Whatcom County Public Works Natural Resources Division has been monitoring 17 sites in the Nooksack River watershed since 1998. Sites are currently sampled twice per month for water temperature, turbidity, and fecal coliform bacteria.

Commercial shellfish beds located on the Lummi Indian Reservation were downgraded from “approved” to “restricted” status in various areas in Portage Bay from 1996 to 2006. The cause of the downgrades was attributed to Nooksack River water entering Portage Bay (Ecology 2000). According to the 1997 DOH Sanitary Survey of Portage Bay, fecal contamination of the Nooksack River was the result of manure management practices by dairy farms in the Nooksack River watershed, and these sources represent a high probability of being the principal source of fecal contamination in Portage Bay. The presence of Nooksack River water in Portage Bay occurs frequently and is evidenced by lowered salinities, salinity-based stratification, and/or color. In general, elevated fecal coliform bacteria levels in Portage Bay are associated with lower surface salinities.

In 2000, a Total Maximum Daily Load (TMDL) for the Nooksack River was developed (Ecology 2000) and a TMDL implementation plan was executed (Ecology 2002). Improvement in water quality that resulted in the reopening of shellfish beds in 2006 was generally attributed to the combined effects of inter-agency coordination; water quality monitoring in Portage Bay and the Nooksack River watershed; compliance enforcement inspections by the EPA and Washington State Department of Ecology; and technical assistance and financial support to Nooksack River watershed dairy operations and municipalities (LWRD and Salix 2006). Although these efforts were initially successful in dramatically improving water quality in the Nooksack River watershed (essentially all of the TMDL targets were achieved at all of the quantification sites at

the end of the first quarter in 2004) and the reopening of all of the Portage Bay shellfish growing area, soon after the shellfish beds were reopened these improvements started to be reversed. The degradation trends of water quality in the Nooksack River and Portage Bay was obvious as early as 2010. In September 2014, a 335-acre portion of the Portage Bay shellfish growing area was voluntarily closed to harvest by the Lummi Nation to protect public health after two monitoring sites exceeded the NSSP fecal coliform standards. In March 2015, after poor water quality was encountered again in November 2014 that affected additional sample sites, the DOH changed the classification of nearly 500 acres of Portage Bay, including the portions already under the voluntary closure, from “approved” to “conditionally approved.” The conditional closure prohibits commercial shellfish harvest from April through June and from October through December (DOH 2015). Due to the poor water quality and associated public health threat, the Lummi Nation has also closed these areas to ceremonial and subsistence harvests.

The primary goal of the Nooksack River Watershed MST Study is to identify sources of the fecal coliform contamination in the Nooksack River and Portage Bay. Information regarding the sources (human, ruminant, or other) of the fecal coliform contamination will aid in identification of strategies to reduce bacterial loading in the Nooksack River and Portage Bay. Potential sources of contamination are failing septic systems (human-source), farms with poor manure management (ruminant-source), ruminant-type wildlife (ruminant-source) and/or non-ruminant wildlife including birds and marine mammals (other non-human, non-ruminant sources).

3.1.1 Objectives and Goals

The Nooksack River Watershed MST Study will provide information regarding the types of fecal coliform contamination at selected sample sites in the Nooksack River, its tributaries, and Portage Bay. Potential sources of fecal coliform contamination include human, ruminant, or other (*e.g.*, non-ruminant wildlife, including birds or marine mammals).

Additional studies may be needed to further refine sources, depending on the results of the Nooksack River Watershed MST Study. Results will inform collaborators on the Nooksack River TMDL implementation and the Whatcom Clean Water Program on the selection of strategies for reducing fecal coliform loads in the Nooksack River and Portage Bay, with the ultimate goal of meeting Nooksack River TMDL targets and Washington State, Lummi Nation, and NSSP water quality standards.

The EPA Region 10 Manchester Laboratory will assist the Nooksack River Watershed MST Study partners (LWRD and Whatcom County) by providing the described MST analyses, with EPA lab staff funding and support provided by the EPA Region 10 Office of Water and Watersheds.

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4. PROJECT DESCRIPTION (A6)

4.1 Partners

The Nooksack River Watershed MST Study is a partnership between the Lummi Nation Water Resources Division (LWRD), Whatcom County Public Works Natural Resources Division (Whatcom County), and EPA Region 10.

4.2 Project/Task Description and Schedule (A6)

This QAPP provides information regarding the collection and analysis of water samples from the Nooksack River, its tributaries, and Portage Bay to help determine the source of fecal coliform contamination in these waterbodies. The basic field and analytical tasks required to achieve the objectives of this project are:

1. Collect grab samples of water from designated locations within the watershed.
2. Analyze the samples for the presence of species-specific *Bacteroides* markers using polymerase chain reaction (PCR) technology.

This Nooksack River Watershed MST Study will begin in January 2016 and is expected to be completed in April 2017. Samples will be collected from eight sites in the Nooksack River watershed and Portage Bay for twelve months over the January 2016 to December 2016 period. Laboratory analysis may continue for up to four months following receipt of the final batch of samples. The final laboratory results will be provided to all partners by March 31, 2017. Table 4.1 includes a schedule for conducting tasks related to this project. The schedule is a guideline only as it is possible that unforeseen circumstances and conditions will require adjustment to some or all of the following proposed dates. A sampling schedule with specific dates and any subsequent changes will be communicated to the EPA lab staff and the EPA RSCC prior to sample collection.

Table 4.1 Timeline for the Nooksack River Watershed MST Study

Task	2016												2017				
	D	J	F	M	A	M	J	J	A	S	O	N	D	J	F	M	A
QAPP Approval	X																
Sample Collection		X	X	X	X	X	X	X	X	X	X	X	X				
Lab Analysis		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	
Final Lab Reporting																X	X

4.3 Data Quality Objectives and Criteria (A7)

The primary data quality objective for the Nooksack River Watershed MST Study is to characterize the qualitative (presence/absence) source(s) of fecal contamination at the targeted locations to the following level of differentiation: human source, ruminant source, or other (non-human, non-ruminant) source. The results will be used to determine if additional source identification work is needed at these locations, and will help guide development of strategies to reduce bacterial loading in the Nooksack River and Portage Bay.

The microbial source tracking analytical methodology is not quantitative; laboratory analysis results indicate either the presence or absence of human source, ruminant source, or other *Bacteroides* species. Due to the qualitative results of the laboratory analysis, the precision and accuracy of the method is unknown at this time and information about the relative contribution of the various sources in each sample will not be possible to develop.

Accuracy is a measure of the closeness of a sample analysis result to the “true” value. Although the accuracy of the MST method overall is currently unknown, the accuracy of the data will be determined through the use of positive and negative laboratory QA/QC samples and the calculation of specificity (see Sections 6.6 and 6.6.6).

Representativeness is defined as the degree to which data accurately and precisely represents characteristics of a population, parameter variations at a sampling point, or an environmental condition. For this project, representativeness is ensured through the collection of samples at representative locations of the sampled waterbody. Collection and analysis of duplicate field samples provides information about the field variability of bacterial sources.

Data are comparable if collection techniques, measurement procedures, methods, and reporting units are equivalent for the samples within the study. Comparable data for this project will be obtained by following field and laboratory methods as specified in this QAPP and specifying standard units for results. All samples will be collected on the same day to ensure comparability.

Analytical completeness is defined as the percentage of valid analytical results requested. Although collection and analysis of 100% of the samples planned for sampling and analysis in this QAPP is anticipated, sample collection and analysis is considered complete if 90% of planned samples are collected and analyzed.

The desired method sensitivity is one strand of *Bacteroides* DNA targeted during PCR. It is anticipated that the presence of one strand of DNA in a sample will be detected, and is reported as a “presence” of the appropriate source type (human, ruminant, other).

The measurement performance criteria/acceptance criteria for this project are discussed in Section 6, Quality Control. In general, if a sample, or associated controls, fall outside of the acceptance criteria, they are rejected and either re-sampled or re-analyzed, as appropriate.

4.4 Special Training and Certification (A8)

Field personnel who are responsible for the collection of Nooksack River Watershed MST Study samples are trained in aseptic sampling techniques. Supervisors are responsible for ensuring that staff members are adequately trained. No special training or certification is required.

Although the MST analysis is not an accredited parameter, the EPA Region 10 Laboratory's Quality System is accredited by the NELAC Institute.

4.5 Documents and Records (A9)

4.5.1 QAPP Distribution

The LWRD Water Resources Specialist I is responsible for maintaining and updating the Nooksack River Watershed MST Study QAPP. The LWRD Water Resources Manager is responsible for ensuring that appropriate project personnel have the most current, approved version of the QAPP, including any updates. The final version of the QAPP and any updates will be distributed in portable document file (PDF) format.

Substantial QAPP updates will be transmitted to all partners for approval as an entire document with identification and justification of changes. Major updates will result in a change in the number before the decimal point in the QAPP version number (*e.g.*, change of name from Version 1.0 to 2.0).

Minor updates to the QAPP will be transmitted to all partners for approval via a letter that identifies changes and justifications. Minor updates include correction of mistakes and non-substantial changes to the QAPP. Corrections of mistakes are tracked through the use of a lower case letter at the end of the QAPP version number (*e.g.*, change of name from Version 1.0 to 1.0a). Non-substantial minor changes are tracked through change of the number following the decimal point in the QAPP version number (*e.g.*, change of name from Version 1.0 to 1.1).

4.5.2 Field and Laboratory Documentation and Records

Field documentation includes but is not limited to field notes, photographs, sample datasheets, chain-of-custody forms, and laboratory results. All field documentation will be archived in electronic and paper format by the Nooksack River Watershed MST Study partner collecting the samples.

Laboratory documentation includes but is not limited to raw data, sample preparation and analysis logbooks, and results of calibration and QA/QC checks. The EPA Region 10 Laboratory will archive the following documents: (1) signed hard copies of sampling and chain-of-custody records; and (2) electronic and hard copies of analytical data. The EPA Region 10 Laboratory will store all sample receipt, sample log-in, and laboratory instrument documentation for a minimum of ten years. Laboratory documentation is generated and maintained by the EPA Region 10 Laboratory.

All electronic records are stored on secured servers that are backed up regularly, ideally nightly.

4.5.3 Quarterly and/or Final Reports

The EPA Region 10 Laboratory will provide a final laboratory analysis report to all project partners within four months of the last sampling event (*i.e.*, April 2017). The report will be distributed to all individuals included on the distribution list.

5. DATA GENERATION AND ACQUISITION

The elements in this section ensure that appropriate methods for sampling, measurement and analysis, data collection, data handling, and QA/QC activities are employed and documented.

5.1 Sampling Design (Experimental Design) (B1)

The EPA Region 10 Laboratory will analyze up to 120 MST samples over the course of the project, including quality control samples. Samples will be collected monthly at eight locations for twelve months. Field staff will collect grab samples of approximately 250 ml of water at each designated site. One sterile transfer blank and one field duplicate will be included as quality control samples in each monthly sample batch. The samples will be shipped to the EPA Region 10 Laboratory within the 30-hour maximum holding time. The EPA Region 10 Laboratory will analyze the samples using *Bacteroides* host specific PCR and classify the fecal sources as human, ruminant, other (non-human, non-ruminant), or absent.

The sampling locations are listed in Table 5.1 and are shown in Figure 5.1. The sites were chosen to provide a wide spatial distribution of sampling efforts. Sites represent upstream (3) and downstream (2) reaches of the Nooksack River, five tributaries to the Nooksack River (4-8), and Portage Bay (1).

The sampling period was selected to provide dry and wet season sampling events. No storm-specific sampling is planned as part of this project but it is possible that a sampling event will coincide with a storm event.

Table 5.1 Sample Locations for MST Study

#	Location	Lat	Long	Site ID	Agency
1	Portage Bay	48.72375	-122.64470	DH050	LWRD
2	Lower Nooksack River at Marine Drive Bridge	48.79068	-122.59070	SW118	LWRD
3	Upper Nooksack River at Everson	48.91788	-122.348296	M5	Whatcom County
4	Bertrand Creek	48.924333	-122.528832	B1	Whatcom County
5	Fishtrap Creek	48.914081	-122.519835	F1	Whatcom County
6	Kamm Creek	48.945265	-122.440334	K1	Whatcom County
7	Scott Ditch	48.919109	-122.462896	S1	Whatcom County
8	Tenmile Creek	48.853635	-122.572188	T1	Whatcom County

Datum for coordinates: North American High Accuracy Reference Network of 1983 (North American 1983 HARN)

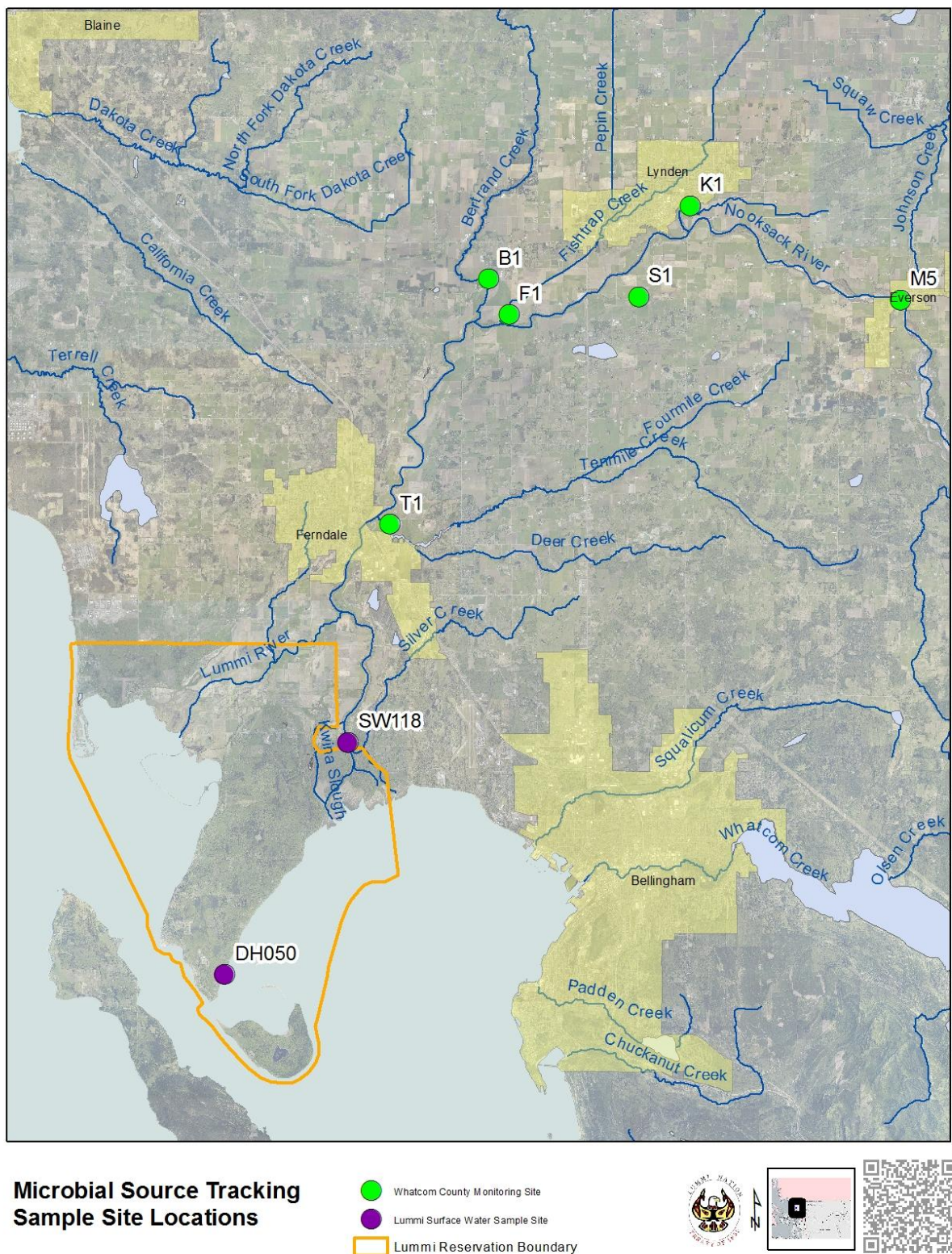


Figure 5.1 Nooksack River Watershed Microbial Source Tracking Study Sample Site Locations

If sample sites become inaccessible, a nearby, safely accessible, representative location may be sampled instead. Sampling at a location different from the designated sample site will be noted on field datasheets and chain-of-custody forms. Partners will be informed of sampling at a different, representative location.

5.1.1 Sampling Schedule

All sites will be sampled on the same day pursuant to an existing coordinated effort among the partners to characterize the water quality and bacterial load distribution in the Nooksack River watershed on a monthly basis. Sample collection is targeted for the day prior to NSSP sampling of Portage Bay. Sampling in Portage Bay for the NSSP is scheduled by the DOH and conducted by the LWRD and DOH on alternate months. Sampling of sites will be scheduled in coordination with the EPA Region 10 Laboratory in 3-6 month intervals to the extent practicable.

Sampling of sites is scheduled for a Monday, Tuesday, or Wednesday to ensure that the EPA Region 10 Laboratory receives the samples no later than Thursday of the sampling week. Sites are sampled randomly, in the sense that season and weather are not used to stratify sampling efforts. Sampling is restricted by practical considerations, including requirements for a sufficient tidal elevation to access marine sites in Portage Bay.

Appendix D provides a summary schedule for field duplicate sample collection and responsibility of sterile field transfer blank sample preparation.

5.1.2 Health and Safety

During MST sample collection, field personnel are exposed to water that is likely to be contaminated with bacteria and other pathogens. Although coliforms are not usually pathogenic themselves, their presence is an indicator of potential pathogenic bacterial or viral contamination. It is expected that field personnel will use good hygiene and good sense in undertaking sampling activities. Powder-free rubber or latex gloves are available for use. Hands are washed thoroughly with soap and water or appropriate sanitary wipes as soon as possible after sampling at sites that may be contaminated with biological pathogens, and again prior to eating or drinking. Sampling equipment are cleaned and dried after use.

No water quality measurement is worth risking injury or death. Field personnel must be aware of the environment, use common sense and training, and not exceed their abilities or limits. Field work is never conducted alone. All LWRD *Health and Safety Plan* (LWRD 2015) or Whatcom County safety requirements and guidelines will be followed at all times while conducting fieldwork.

5.2 Sample Identification

Sampling locations and site identifications have been assigned by the Nooksack River Watershed MST Study partners. Each sample collected and analyzed by the EPA Region 10 Laboratory will be assigned a unique laboratory sample number. Laboratory sample numbers will be assigned in blocks of 50 by the EPA Regional Sample Control Coordinator. Each sample

sent to the EPA Laboratory must include the assigned project code and the unique laboratory sample number in the format YYWWXXXX where:

YY: Calendar year in which samples were collected.

WW: Calendar week number in which samples were collected.

XXXX: Laboratory identification number assigned by the EPA Regional Sample Control Coordinator. This number is unique to each sample.

It is recommended that while the sample week number will change, the laboratory identification numbers are used consecutively through the assigned block in order to avoid duplication at the laboratory. For example, if one event in week 33 of 2015 ended at 15334933 the next event in week 37 of 2015 would use 15374934.

Nooksack River Watershed MST Study partners will coordinate to ensure that laboratory sample numbers are properly assigned and the sample location identifications for each unique laboratory sample number are tracked. The LWRD Water Resources Specialist I and Whatcom County Senior Planner will be responsible for ensuring proper sample tracking and numbering.

Prior to collecting a sample, the MST bottles are labeled with the following information:

- Region 10 Project Code
- Region 10 Sample ID (the unique laboratory sample number described above)
- Sample collection date and time
- Laboratory analysis requested

5.3 Sampling Methods (B2)

5.3.1 Sample Bottles

Sterile, plastic, 250-ml sample bottles are provided by the EPA Region 10 Laboratory. Bottles are inspected by field personnel upon receipt, and any damaged bottles or bottles with caps removed are returned to the EPA Laboratory. Only undamaged, tightly capped bottles with intact foil cap are used to collect MST samples.

5.3.2 Sample Collection and Handling

The samples will be collected following guidelines as cited in *Microbiological Methods for Monitoring the Environment: Water and Wastes* (EPA 1978). Sampling methods may vary slightly between standard operating procedures used by samplers and different conditions encountered in the field. The following is general guidance for the samplers.

When arriving at a sample site, determine where the MST sample will be collected based upon visual assessment of the waterbody and previous experience. MST sample sites are visited monthly by study partners as part of coordinated water quality monitoring of the Nooksack River watershed. Upon arrival at the sample site:

1. Determine the path of sample collection in the waterbody prior to physically collecting the sample. The sample is collected at a representative location (Section 5.3.3).
2. Minimize disturbance to waterbody (Section 5.3.4).
3. Use a sampling wand to collect the MST sample unless unsafe or impractical. If site conditions prevent sampling using the wand (*e.g.*, very shallow water), the MST sample can be collected by hand.
4. Sample upstream of any influences of the sampler.
 - a. When sampling from a boat, sample water from near the bow (front of the boat) while slowly moving forward over water that the boat has not previously come into contact with during the sample run.
 - b. Sample outside of the zone of influence of the sampler(s) if the sample site requires wading. Water and sediments can be entrained by the movement of the sampler.

5.3.3 Representative Location

MST samples are collected at a representative location. The following are considered when selecting a representative location at the sampling site:

- Avoid areas along margins, where debris accumulates, and other areas that are not characteristic of the waterbody at the sample site.
- Select an area that minimizes disturbance to the waterbody (Section 5.3.4).
- For wading sites, unless safety precludes wading into the water, avoid collecting samples along the shoreline where waves are breaking and washing across the beach.
 - Sample seaward of debris and seaweed generally found in the water close to the shoreline.
 - Avoid areas of entrained air in the wave-wash zone.
- If a representative location cannot be found, use professional judgment to determine whether the site should be sampled. Note observations of non-representativeness and explanation of rationale for choosing to collect or not collect a sample.

5.3.4 Site Disturbance

Ensure that the sample site is not disturbed prior to MST sample collection. A site is disturbed if sediments or other materials (*e.g.*, plants, benthic algae) settled at the bed of the waterbody are suspended into the water column, or debris falls into the water at the sample site. Fecal coliform bacteria in bottom sediments can remain viable for many weeks. Disturbing sediments can re-suspend these bacteria and result in temporary uncharacteristically high bacteria concentrations and may bias MST results. Strategies for avoiding site disturbance include:

- Avoid walking in the waterbody or near the edge of the waterbody.
- If wading into the waterbody is required, approach the sample site from the downstream side.

- Use a wand to collect samples.

Occasionally, it will be necessary to collect a second MST sample (e.g., if the first attempt at sampling is unsuccessful). If the site was not disturbed by the first attempt at sample collection, the second attempt at collecting a MST sample can follow immediately after the first with a new, sterile bottle. If the area appears disrupted by the first sample collection attempt, consider the following when attempting to re-sample:

1. Subsequent samples should be at a different, but representative location at the sample site.
2. If overall conditions at the sample site are stable, the disturbance will dissipate within a few minutes, and if water quality is not likely to change from when sampling started, the disturbed location may be re-sampled after the disturbance has passed.
3. If subsequent samples cannot be relocated or sampled within a few minutes, consider a complete re-sampling of the site at a later time the same day.

5.3.5 Sample Collection With a Sampling Wand

Use a sampling wand for MST sample collection unless impractical or unsafe.

1. Do not rinse the sample bottle.
2. Label closed (never opened) and undamaged laboratory-supplied sample bottle as described in Section 5.2. Do not write directly on the sample bottle. [Note: masking tape works well as a label as it adheres nicely to the bottle and can be removed in the laboratory without leaving glue residue behind.]
3. Attach capped sample bottle to wand. The top of the sample bottle should be several inches upstream/up-gradient of every part of the sample wand (i.e., no sample water will touch any part of the wand before flowing into the sample bottle).
4. Open sample container. Do not place bottle or cap on ground and do not touch or allow any foreign materials to come into contact with bottle opening or threads, or the inside of the bottle cap. Do not set the cap down; hold the lid carefully touching the outside only.
5. Vertically dip the sample bottle, opening first, into the water column and then in one motion, rotate the bottle in the direction of the current (upstream), so that trapped air can escape as the bottle fills in an upstream/up-gradient arc.
 - a. The bottle should be no deeper than 6 inches below the water surface.
 - b. The water from the surface should not enter the bottle, unless low flows prevent sampling of water below the surface.
 - c. Ensure that bottle opening is always upstream of the wand.
6. Continue the motion until the bottle is nearly full, then rotate the bottle to remove it vertically from the waterbody.
7. There should be a 1 inch head space in the neck of the bottle, to allow adequate mixing by the analyst. If, however, the sample container is overfilled, DO NOT pour out any

excess sample. Place the cap securely on the sample bottle and return it to the analyst overfilled.

8. Carefully recap the sample bottle securely, leaving the foil cap in place. Place sample upright in cooler with bagged ice or cold packs. If bottle cannot be placed on ice immediately after collection, place bottle in sheltered area out of direct sunlight and place in cooler as soon as possible.
9. If the sample is not collected successfully, place an "X" on the label and collect another sample using a new, sterile bottle. Do not collect sample from waters that were disturbed during collection of the unsuccessful sample. See Section 5.3.4 for details about re-sampling at a potentially disturbed site.

5.3.6 Sample Collection by Hand (Without a Sampling Wand)

MST samples can be collected by hand if use of the sampling wand is unsafe or impractical. Very low flows can result in the sampling wand disturbing the bottom of the waterbody during sample collection. The MST sample is collected by hand as follows:

1. Do not rinse the sample bottle.
2. Label closed (never opened) and undamaged laboratory-supplied sample bottle as described in Section 5.2. Do not write directly on the sample bottle. [Note: masking tape works well as a label as it adheres nicely to the bottle and can be removed in the laboratory without leaving glue residue behind.]
3. Rinse hands with distilled or deionized water and dry. Wear powder-free nitrile/latex/rubber gloves and consider the use of safety glasses.
4. Hold the capped sample bottle near base with hand. The top of the sample bottle should be several inches upstream/up-gradient of the sampler's hand (*i.e.*, no sample water will touch the sampler's hand before flowing into the sample bottle).
5. Follow steps 4-9 in Section 5.3.5.

5.3.7 Field Duplicates

A field duplicate is collected and submitted with each monthly sample batch. The duplicates are given their own sample number and labeled as "duplicate." The field duplicate sample is factored into the total number of samples (*i.e.*, eight sites plus one duplicate and one field transfer blank for a total of ten samples each month). Appendix D details which site will be duplicated for each monthly sample batch.

5.3.8 Field Transfer Blank

A field transfer blank sample is prepared and submitted with each monthly sample batch. The field transfer blanks are given their own sample number and labeled as "field transfer blank." The field transfer blank sample is factored into the total number of samples (*i.e.*, eight sites plus one duplicate and one field transfer blank for a total of ten samples). Field transfer blanks monitor for the introduction of extraneous material into the samples during field sample

handling, transport, storage, and throughout the MST laboratory analysis process. Appendix D details which partner will be responsible for collecting each field transfer blank.

5.3.9 Corrective Actions

Corrective actions for deficiencies will be addressed immediately in the field. Corrective actions include discarding improperly collected or handled samples and re-sampling. The Nooksack River Watershed MST Study partners will collaborate on solutions to any problems that cannot be remedied in the field, such as site inaccessibility.

5.4 Sample Handling, Custody and Documentation (B3)

The MST samples are stored on ice upon collection and are shipped to the EPA Region 10 Laboratory for analysis within 30 hours of sample collection. The eight MST samples, one field duplicate MST sample, and one field transfer blank are packaged in a polystyrene cooler with ice packs for delivery to the EPA Region 10 Laboratory. The Nooksack River Watershed MST Study partners intend to ship all samples for each monthly sample batch in a single container; however, in the event that this is not possible, this QAPP contains provisions for delivering samples to the EPA Region 10 Laboratory in multiple shipping containers.

The EPA Region 10 Laboratory must receive all samples no later than Thursday of the sampling week to allow sufficient time for filtration and initial processing of the samples without staff overtime or compensatory time charges. Samples are accepted for analysis only if the temperature control measures below 10°C but above freezing at the time the samples are received by EPA Region 10 Laboratory staff. The Regional Sample Control Coordinator and lab staff are notified of the anticipated field sampling schedule prior to collection and are provided with the required shipment information on the day of each sample shipment (*e.g.*, air bill or tracking number, number of samples for analysis, project code, date shipped).

Bottles are labeled with the unique sample site identifier prior to sampling.

An EPA Region 10 chain-of-custody form accompanies the samples, and includes the following for each sample:

- Site location information
- Sample number (the EPA Region 10 laboratory sample number)
- Site description/ID (LWRD, DOH, or Whatcom County sample site ID)
- Date sample collected
- Time sample collected
- EPA Project Code

The chain-of-custody form is signed and dated for relinquishment of custody upon shipment to the EPA Region 10 Laboratory. A copy of the chain-of-custody form is attached as Appendix A.

The LWRD Water Resources Specialist I will compile all samples into one shipping container and prepare the container for overnight shipment via FedEx to the EPA Region 10 Laboratory. The

EPA Region 10 Laboratory will return the re-useable cooler to the LWRD via FedEx. As needed, EPA Region 10 Laboratory will provide new sample bottles and ultra pure water for field transfer blank.

5.5 Analytical Methods (B4)

DNA microbial source tracking is determined by the EPA Region 10 Laboratory using *Bacteroides* host specific polymerase chain reaction (PCR). Samples are filtered, DNA is extracted from the filters, the general *Bacteroides*, human and ruminant primers are used to amplify the target DNA in the samples, and the amplified product is separated using gel electrophoresis. Laboratory microbiologists will interpret the results and report the presence or absence of each source (human, ruminant, and other).

Methods utilized by the EPA Region 10 Laboratory were derived from the PCR method developed by Kate Field at Oregon State University. The EPA Office of Research and Development has developed the methods and work instructions for this analysis. These work instructions (#Mi-WI008, effective date 8/24/2015) are appended to this QAPP and include: Sample Filtration, DNA Extraction, PCR, Gel Electrophoresis, and Reading and Interpretation. It is possible that additional *Bacteroides* host specific primers may be developed to detect other sources during the performance period of this QAPP. If this new technology becomes available, the associated methods and work instructions may be appended to this QAPP. The QA/QC procedures for laboratory analysis are included in Section 6.

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6. QUALITY CONTROL (B5)

Quality assurance/quality control procedures include aseptic field techniques, field transfer blanks, laboratory holding times, chain-of-custody forms, and laboratory QA/QC procedures.

6.1 Field Duplicate

A field duplicate will be collected and submitted with each monthly sample batch. The duplicates will be given their own sample number and labeled “field duplicate” in the chain-of-custody form comments. The location is correctly identified (not masked or blind). The field duplicate sample will be factored into the total number of samples (*i.e.*, eight sites plus one duplicate and one field transfer blank for a total of ten samples per month).

Although the field duplicate is not a QA/QC sample per se, it will provide an indication of variability and may indicate the range of possible sources of fecal contamination.

Selection of sites for duplication is determined by the Nooksack River Watershed MST Study partners in advance (Appendix D).

6.2 Aseptic Field Techniques

Field staff will ensure that all MST samples are collected using sterile techniques. This includes inspecting the laboratory-provided sample bottles for potential contamination prior to use and proper handling of the sample bottle during MST sample collection. In particular, proper handling includes ensuring that:

- The bottle is not contaminated by contact with sampler’s hands, sampling wand, or foreign materials such as plants and substrate in the waterbody.
- The cap is not contaminated by contact with sampler’s hands or foreign materials.

6.3 Field Transfer Blank

A field transfer blank QA/QC sample is supplied to the EPA Region 10 Laboratory for analysis in each sample batch. A sterile bottle containing 100 ml of ultra pure water is provided to the LWRD or Whatcom County by the EPA Region 10 Laboratory. In the field, field personnel will transfer the ultra pure water to a sterile 250 ml sample bottle. The field transfer blank sample is treated like all other samples for the remainder of the field visit, during transportation to the EPA Region 10 Laboratory, and in laboratory procedures. The field transfer blanks will be given their own sample number and labeled “field transfer blank” in the chain-of-custody form comments.

Field transfer blanks monitor for the introduction of extraneous material into the samples during field sample handling, transport, storage. The field transfer blank verifies the ability of field personnel to collect, handle, and transport bacteria samples using aseptic techniques (*i.e.*, without contaminating the sample).

A positive result indicates the presence of contamination most likely due to poor aseptic technique in the field, contact with other samples, or damaged storage containers.

LWRD and Whatcom County field personnel will provide the field transfer blank sample on alternate months (Appendix D).

6.4 Holding Times

The EPA Region 10 Laboratory holding time of 30 hours is observed for sample delivery. If samples are received by the Region 10 Laboratory outside of the maximum holding time, all samples in the batch will be discarded.

6.5 Chain-of-Custody Form

Chain-of-custody forms are used to handle and track samples from field collection to delivery (typically shipped) to the EPA Region 10 Laboratory.

A chain-of-custody form is provided by the EPA Region 10 Laboratory. The chain-of-custody form is filled out while the sampler is in possession of the samples and included inside the sample delivery box. The number on the chain-of-custody form will follow the samples through analysis to final reporting. Multiple chain-of-custody forms will be required if more than one shipping container is to be used.

Copies of chain-of-custody forms are saved in hard copy and electronic format by LWRD and Whatcom County staff. A copy of the chain-of-custody form is included in Appendix A.

6.6 EPA Region 10 Laboratory QA/QC

Laboratory analysis and procedures will comply with the guidelines described in the *Quality Assurance Manual for the U.S. EPA Region 10 Manchester Environmental Laboratory* (EPA Region 10 Laboratory 2015). A copy of this document is available from Stephanie Bailey, EPA Region 10 Laboratory Microbiologist.

The following QA/QC activities are performed by the EPA Region 10 Laboratory performing analytical services in support of this project. Table 6.1 summarizes these activities.

6.6.1 Filtration Blank

The filtration blank is designed to screen for contamination through the screening primer portion of the MST process. False positive results indicate contamination at some point in the laboratory analysis process. False positive results may not disqualify the data if a cause can be determined and if the cause is determined not to impact the remainder of the samples processed in that batch. If the cause cannot be determined or the cause may have impacted the remainder of the samples, the sample batch associated with the filtration blank will be discarded. Filtration blanks are created by the EPA Region 10 Laboratory at time of filtration.

6.6.2 Positive Control

One positive control is prepared with each batch of samples to be amplified. The positive control will serve as the reference sample and consists of plasmid DNA containing the target sequence or the appropriate fecal DNA. The positive control verifies that the PCR amplification (*e.g.*, the polymerase, master mix, templates) and gel electrophoresis systems are functioning properly and that the laboratory analysis process (including gel reading and interpretation) can detect the presence of the positive reference sample. The positive control also verifies that a negative result in an unknown sample is actually negative for that particular primer.

If the positive control is not acceptable or is questionable, the amplification steps will be repeated on the controls' associated batch of samples.

6.6.3 Negative Control

One negative control is prepared with each batch of sample to be amplified. The negative control is prepared by performing PCR with water instead of a template. The negative control verifies that the PCR amplification (*e.g.*, the polymerase, master mix, templates) and gel electrophoresis systems are functioning properly. The negative control verifies that cross contamination is absent throughout the PCR and gel electrophoresis process, and that gel reading and interpretation are adequate.

If the negative control is not acceptable or is questionable, the amplification steps will be repeated on the controls' associated batch of samples.

6.6.4 Negative Sample Result Interpretation

An actual sample will not be considered negative until it has been subjected to replicate analyses using at least five different concentrations of the sample containing the purified DNA.

6.6.5 Out of Specification

Results that are out of specification will be reviewed by the Technical Director for Microbiology and the Project Officer for a decision on whether the data should be included in the report. Method experts in EPA's Office of Research and Development may also be consulted for advice on the quality of the data in these situations.

6.6.6 Specificity

Specificity is the ability of a given MST method to discriminate between various animal sources. Specificity can be calculated from the results of negative QA/QC controls, which can either test negative (true negative) or positive (false positive).

$$\text{Specificity} = \frac{\text{True Negatives}}{(\text{True Negatives} + \text{False Positives})} \times 100\%$$

Although there is currently no consensus, specificity values below 80 percent reflect questionable discriminatory power (EPA 2005).

6.6.7 *Additional Laboratory Checks*

EPA Region 10 Laboratory staff will review all QA/QC results, including negative and positive samples, and documenting requirements. See Appendix C Microbiology Laboratory Data Review/Release Form for details.

Table 6.1 Summary of EPA Region 10 Laboratory QA/QC Procedures

QA/QC Type	Description	Frequency	Corrective actions/ consequences of failure	Method/SOP QC Acceptance Limits	Acceptance Criteria/Measurement Performance Criteria
Positive Control	Reference sample/positive control of plasmid DNA containing target sequence or the appropriate fecal DNA	One per PCR batch, each analysis/primer	Repeat amplification step or gel electrophoresis; data reviewed, may or may not disqualify data depending on cause	Positive reaction, DNA present	Appropriate DNA amplification
Duplicate	Field duplicate indicates field variability and laboratory precision	One per sampling event; analyzed as separate sample	Data reviewed, may or may not disqualify data depending on cause	Same result	Appropriate DNA amplification
Filtration Blank	Negative filtration control using sterile rinse water; assessing entire process	One per filtration series per day	Data reviewed, may or may not disqualify data depending on cause	Negative, no DNA	Negative, no DNA
Negative Control	PCR with ultra pure water instead of template	One per PCR batch, each analysis/primer	Repeat amplification step or gel electrophoresis; data reviewed, may or may not disqualify data depending on cause	Negative, no DNA	Negative, no DNA
Field Transfer Blank	Field transfer blank using ultra pure water; used as a negative extraction control	One per sampling event; analyzed as a separate sample	Data reviewed, may or may not disqualify data depending on cause	Negative, no DNA	Negative, no DNA

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7. INSTRUMENTS/EQUIPMENT

7.1 Instrument/Equipment Testing, Inspection, and Maintenance (B6)

All field sampling equipment are kept clean and in working order.

Laboratory instruments and equipment (*e.g.*, waterbaths, incubators, thermal cyclers, and other equipment required by the applicable analytical methods) are tested, inspected, and maintained as required by the laboratory standard operating procedures (SOPs) and manufacturer's instructions. Laboratory instrument and equipment testing, inspection, and maintenance records are maintained by the EPA Region 10 Laboratory.

7.2 Instrument/Equipment Calibration and Frequency (B7)

No field instruments that require calibration are used for the MST Study.

Laboratory instruments and equipment (*e.g.*, pH meters, waterbaths, incubators) are calibrated as required by the laboratory SOPs and manufacturer's instructions. Laboratory instrument and equipment calibration records are maintained by the EPA Region 10 Laboratory.

7.3 Inspection/Acceptance Requirements for Supplies and Consumables (B8)

Consumable supplies used in the field will consist primarily of sterile, 250-ml plastic sample bottles. The quality of consumable supplies such as sample bottles used for this project should be documented by the supplier and certificates should be available on request. Bottles are provided by the EPA Region 10 Laboratory and are batch tested for sterility prior to sending them to the field for sample collection. Bottles other than those prepared at the EPA Region 10 Laboratory will not be acceptable for use in the MST Study. Sample bottles are inspected by LWRD and Whatcom County field personnel prior to use as described in Section 5.3.1.

All supplies and consumables used in the EPA Region 10 Laboratory are maintained, inspected, and monitored according to the laboratory's work instructions, SOPs, QAPPs, and NELAC requirements.

7.4 Data Acquisition Requirements (Non-Direct Measurements) (B9)

No non-direct measurement or data acquisition are anticipated for the MST Study.

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8. DATA MANAGEMENT (B10)

Field notes for each site will be recorded in a field datasheet, and include the following:

- Sampler(s) name(s)
- Sample number (the EPA Region 10 laboratory sample number)
- Site description/ID (the internal LWRD, DOH, or Whatcom County sample site ID)
- Date sample collected
- Time sample collected
- Notes regarding human or animal activity in the area at the time of the sampling, if available
- Additional observations
- EPA Project Code

Sampling activities performed by the LWRD are documented in the LWRD Water Database. The LWRD Water Database is an Access-based database maintained by the LNR Database Manager and stored on secure servers that are backed up nightly. Sampling activities performed by Whatcom County are documented in field datasheets or electronic format.

Hard copies of field datasheets, chain-of-custody forms, and laboratory results are scanned and saved in electronic format on secure servers by each partner organization. Hard copies are archived and managed by each partner organization.

In addition, the EPA Region 10 Laboratory maintains a logbook that includes the information provided in the chain-of-custody forms, as well as time of analysis and analyst initials. Quality control results are recorded on bench sheets. All data generated by the EPA Region 10 Laboratory are subject to a peer review then signed-off by the Microbiology Team Technical Director. Sample custodian staff process and distribute all information and documentation in accordance with laboratory SOPs. Logbooks, bench sheets and final reports are stored on-site. All data generated during this project are processed, stored, and distributed according to laboratory SOPs.

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9. ASSESSMENT AND OVERSIGHT

9.1 Assessments/Oversight and Response Actions (C1)

The LWRD Water Resources Specialist I and the Whatcom County Senior Planner are responsible for oversight of field sample collection and QA/QC procedures (*e.g.*, field transfer blank preparation, holding times, chain-of-custody forms). Corrective actions for deficiencies will be addressed immediately in the field or will be resolved through collaboration of the Nooksack River Watershed MST Study partners.

The EPA Region 10 Laboratory routinely performs performance checks using method-specific positive and negative controls. An internal assessment of the data and results is also routinely conducted by the Laboratory QA Coordinator.

Corrective actions will be implemented in response to any QA/QC results or detection of unacceptable data. These corrective actions will be developed in consultation with the EPA Office of Research and Development, keeping the partners informed of any impacts on the data. If required, corrective actions will be documented and approved by the EPA QA Manager.

9.2 Reports to Management (C2)

If, for any reason, the schedules or procedures provided in the QAPP cannot be followed, the Nooksack River Watershed MST Study partners will work with EPA Region 10 and Laboratory staff to make modifications to the schedule or procedures. The changes will be reviewed and approved by the QA Officer. All parties in the distribution list will be given a copy of the QA Officer-approved changes for reference and for the project file.

A final laboratory report will be generated by the Region 10 Laboratory at the completion of the project, within four months of receipt of the last samples for analysis. This report will include a discussion of the findings, interpretation of data and an executive summary. The report will be provided to all individuals listed in the distribution list. In addition, short summary reports will be provided during the study period to keep those on the distribution list informed of the progress of the project and to allow for adaptations in the sampling program. Difficulties that may result in delayed data and reporting will be communicated to those parties on the distribution list.

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10. DATA REVIEW AND USABILITY

10.1 Data Review, Verification, and Validation (D1, D2)

Data review, verification, and validation are largely the responsibility of the EPA Region 10 Laboratory. The final report will only include data that have been reviewed and verified as reliable by the EPA Region 10 Laboratory, and will indicate the quality of all data reported.

10.1.1 Data Review

EPA Region 10 data verification and peer review will be accomplished following the EPA Region 10 Laboratory SOP for data review (Mi_D001A). An example Microbiology Laboratory Data Review/Release Form is included as Appendix C for details of data review checks conducted before release. Data will be qualified as necessary to convey to the user any important information that needs to be considered in its use.

10.1.2 Data Verification

Data verification will include a review of the findings of all QA/QC assessment activities including:

- Appropriate sample collection and preparation of field transfer blank sample: assessed during sample collection by responsible field personnel.
- Chain-of-custody procedures: assessed by the responsible field personnel and laboratory sample custodians for EPA Region 10 Laboratory.
- Analytical data collection, recording, and reporting including laboratory QA/QC procedures: assessed by EPA Region 10 Laboratory staff.

Verification of the EPA Region 10 Laboratory analytical results is the responsibility of the Microbiology Technical Director, as required by the laboratory's QA Manual. If any deviations are identified, the potential impact of those deviations on the reliability of the data will be assessed, and the information will be provided to the Nooksack River Watershed MST Study partners through a QA Memo and appropriate flagging of the data.

10.1.3 Data Validation

Data validation is an evaluation of the technical usability of the verified data with respect to the planned objectives of the project. This is accomplished by applying a defined set of performance criteria to the body of data in the evaluation process.

Data validation will evaluate all individual samples collected and analyzed to determine if the results are within acceptable limits. Quantitative or qualitative limits of acceptability are defined for precision, accuracy, representativeness, comparability, and completeness. Typically, data that are reviewed and verified as meeting appropriate standards, including passing QA/QC protocols, by the EPA Region 10 Laboratory will be considered valid for use in final reporting.

10.2 Reconciliation with User Requirements (D3)

All data and related information obtained during the course of this project will be included in a final laboratory report prepared by the EPA Region 10 Laboratory. Presentations of data and data analysis may be made to relevant user groups upon request. All data will include metadata, including any associated qualifiers.

11. REFERENCES

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12. ACRONYMS AND ABBREVIATIONS

COC	Chain of Custody
DOH	Washington State Department of Health
EPA	Environmental Protection Agency
LNR	Lummi Natural Resources
LWRD	Lummi Water Resources Division
MEL	EPA R10 Manchester Environmental Laboratory
MST	Microbial Source Tracking
NSSP	National Shellfish Sanitation Program
PCR	Polymerase Chain Reaction
QAPP	Quality Assurance Project Plan
QA	Quality Assurance
QC	Quality Control
QA/QC	Quality Assurance/Quality Control
RSCC	Regional Sample Control Coordinator (EPA)
SOP	Standard Operating Procedure
TMDL	Total Maximum Daily Load

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13. APPENDICES

Appendix A: Chain-of-Custody Form

Revision 1

DOI: 10.1002/for

Appendix B:

EPA Region 10 Laboratory's Laboratory Wide Procedure

Document # Mi-WI008 for Microbial Source Tracking (MST)

Work Instructions

	LABORATORY WIDE PROCEDURE U.S. Environmental Protection Agency Region 10 Laboratory	Document #: Mi-WI008	Version #: 1
			Page 1
	Title: Microbial Source Tracking (MST) Work Instruction		Effective Date: 8/24/2015

Filtration (Lab 28)

Materials Needed:

- 1) Gloves
- 2) Sterilized filtration funnels
- 3) Vacuum source and manifold to accommodate the filters
- 4) Filter forceps
- 5) 95% non-denatured ethanol
- 6) On-demand Bunsen burner
- 7) Sterilized rinse water
- 8) Supor-200® membrane filters, 0.2 µm pore size, 47 mm diameter (Pall)
- 9) MP Biomedical® Lysing Matrix A, 2 mL tubes

Sample Handling:

Samples should be held between 1-10°C and shipped overnight for filtration within 30 hours of collection.

Controls:


- 1) One filtration control for each batch of samples.
- 2) One transfer blank for each sampling event.

Safety Measures:

- 1) The use of personal protective equipment (lab coat, nitrile gloves and safety glasses) is required.
- 2) Special care should be taken when flame sterilizing the filtration funnels and forceps. To reduce the risk of unintended fire only an on-demand Bunsen burner will be used.

Protocol:

- 1) Label each Lysing Matrix A tube with the sample number it will be associated with.
- 2) Place sterile filtration funnel on the manifold. Aseptically place a Supor® 200 filter on the funnel base (grid side facing up).
- 3) Select a sample and its' associated 2ml labeled tube. Thoroughly shake the sample bottle then pour 100mls of sample into the funnel and apply vacuum. Maintain a vacuum until all of the sample has passed through the filter then rinse the funnel with sterilized Milli-Q water.
- 4) Lift the funnel from the base and using flame-sterilized forceps carefully roll the filter into a small cylinder, gently bending at a 90° angle to prevent the filter from unrolling.
- 5) Aseptically insert the filter into its' associated 2 mL labeled tube.
- 6) Flame sterilize the filtration unit using alcohol. Thoroughly rinse the filtration unit using sterile rinse water prior to moving on to the next sample.
- 7) After the last sample has been filtered, place the labeled tubes in the freezer, lab 113.

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Extraction:
Fast DNA® Kit
(Lab 113)


Materials Needed:

- 1) Tubes containing frozen filters from the filtration process
- 2) Fast DNA® Kit
- 3) DNase/RNase-free 2 mL tubes
- 4) DNase/RNase-free Water
- 5) Teeny Tuff Tags®
- 6) DNase/RNase-free 0.65µL pop-cap tubes

Protocol:


- 1) Pull filters from freezer and select samples to be extracted
- 2) Add 800µL CLS-VF and 200µL PPS to each sample tube
- 3) Place tubes in the bead beater and run for two, 15 second intervals
- 4) Centrifuge sample tubes at 14,000xg for 5 minutes
- 5) Place an appropriate number of new 2mL tubes in a rack and label with the corresponding sample numbers of those tubes containing the filters
- 6) Fill a separate 2mL tube with DNase/RNase-free water and place in the hot block to warm (set to 60°C)
- 7) Add 600µL of well shaken binding matrix to each newly labeled 2mL tube
- 8) Once centrifugation is complete, gently remove the tubes and place upright in a tube rack.
- 9) For each tube containing a filter, carefully withdraw 600µL of sample and place in the corresponding 2mL tube containing the binding matrix. *Do not withdraw any of the bead pellets with the sample.*
- 10) Gently rock tubes back and forth for 5 minutes
- 11) Centrifuge at 14,000xg for 2 minutes
- 12) Open each tube one at a time and gently pour off the liquid into the sink, taking care to prevent pellet loss.
- 13) Place each tube back into the rack. At this point the tubes should only contain pellet; DNA is now attached to the binding matrix.
- 14) To each tube of pellet add 400µL of SEWS-M with ethanol (comes as a concentrate, ethanol must be added before initial use)
- 15) Cap and vortex each tube
- 16) Centrifuge at 14,000xg for 2 minutes
- 17) Pour off liquid and place tubes back in rack. Again add 400µL SEWS-M with ethanol.
- 18) Repeat vortex and centrifuge steps
- 19) Pour off liquid and place tubes in rack. To each tube add 600µL DNase/RNase-free water.

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- Note:** Each tube is rinsed a *total* of 3 times: 2 ethanol rinses followed by a final 600µL DNase/RNase-free water rinse
- 20) Repeat vortex and centrifuge steps
 - 21) Pour off liquid and place tubes in rack, leaving the caps *off* of the tubes
 - 22) Place the opened tubes into the hot block and dry until very little liquid remains
 - 23) While drying, label Teeny Tuff Tags® with the appropriate sample numbers. Place the labels on the 0.65µL pop-cap tubes
 - 24) After each sample has dried add 100µL of the warmed DNase/RNase-free water
 - 25) Vortex well to disrupt the pellet and place back into the hot block for 5 minutes
 - 26) Remove the tubes from the hot block and place in centrifuge. Spin at 14,000xg for 5 minutes
 - 27) Carefully remove the tubes from the centrifuge and place in rack, matching the 2mL sample tubes to their corresponding 0.65µL pop-cap tubes
 - 28) Carefully draw off the liquid from each tube and transfer to the 0.65µL pop-cap tube, being sure to leave the pellet behind. Approximately 100µL of sample will be drawn off
 - 29) At this point the 0.65ul labeled tubes containing the sample extract can be placed in the freezer to safely await the PCR step.

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Master Mix/Tray Set Up (Lab 112)

Reagents:

10X Ex Taq Buffer
 dNTP
 25% Acetamide (0.25g Acetamide/1mL DNase/RNase free water)
 Ex Taq (enzyme)
 Reverse Primer (708R)

- 1) Pull reagents from freezer as well as the forward primer to be run (32F, CF 193, HF 183, or HF 134)
- 2) Load one frozen tray with as many PCR tubes as samples being run (i.e. 5 samples, 5 PCR tubes) Set up desired order/sample location. This is called the dilution tray.
- 3) Load a second frozen tray with PCR tubes that will be used to contain the 5 dilutions of each sample, as well as a positive and a negative control. This is called the PCR tray. On a PCR sample location template note where each sample is going to be located, including the positive and negative controls (see Attachment 2). When trays are loaded proceed to hood. Fill one 2 mL tube with DNase/RNase free water and label accordingly. Label a second 2 mL tube as "Master Mix".
- 4) To each tube in the *dilution* tray, add 9 μ L of DNase/RNase free water.
 To the master mix tube add appropriate amount of DNase/RNase free water (see Attachment 1). Continue adding the rest of the reagents in their appropriate order and amounts to the master mix tube. Order addition will be: DNase/RNase free water, 10 Ex Taq Buffer, dNTP, 25% Acetamide, 708R and the desired forward primer, Ex Taq enzyme. *Note: vortex well each reagent EXCEPT the Ex Taq enzyme; do not vortex.*
- 5) Close the master mix tube and vortex. Place reagents back in the freezer.
- 6) To the *PCR* tray add the following volumes of master mix to their corresponding sample tubes:
 - 1:10 = 24 μ L
 - 1:5 = 23 μ L
 - 1:2 = 20 μ L
 - 1.0 = 24 μ L
 - 2.0 = 23 μ L
 - Negative control = 24 μ L and 1 μ L DNase/RNase free water
 - Positive control = 24 μ L
- 7) Place trays, PCR sample location template, left over master mix and DNase/RNase free water in pass through.


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Polymerase Chain Reaction (PCR) (Lab 113)

- 1) Pull from freezer extracts to be run along with their associated positive control
- 2) Place extracts in small centrifuge and spin to knock any beads down
- 3) Finish filling out the sample location template (see Attachment 2)
- 4) To the PCR tray add the following volumes:
 - a. 1:10 = (24 µL and) 1 µL of the 1:10 dilution sample (tube found in dilution tray)
 - b. 1:5 = (23 µL and) 2 µL of the 1:10 dilution sample (tube found in dilution tray)
 - c. 1:2 = (20 µL and) 5 µL of the 1:10 dilution sample (tube found in dilution tray)
 - d. 1.0 = (24 µL and) 1 µL of the straight extract
 - e. 2.0 = (23 µL and) 2 µL of the straight extract
 - f. Positive control = (24 µL and) 1 µL positive control (not primer but positive)
- 5) All volumes should now be 25 µL in each PCR tube in the PCR tray
- 6) Repeat the above sample additions with each sample extract
- 7) Once filled, close lids
- 8) Turn on thermocycler
- 9) Place a top tray into the quad to be used (found in the drawer immediately below the thermocycler)
- 10) Load quad tray with PCR tubes, being sure sample isn't clinging to side of tubes
- 11) Once quad is loaded, hit "proceed", then left or right arrow until the desired run is selective (i.e. RUM, HUM, GB) then hit "proceed" again. Hit block key until desired block is highlighted then hit "proceed". Select "yes" for heated lid then "proceed" again
- 12) *If running more than one block, start the run of the first block then program the second block by pushing the "block" key to go back to the main menu and then set up the next run.*
- 13) The run takes approximately 2.5 hours
- 14) Label Teeny Tuff Tags® with appropriate sample numbers
- 15) When thermocycler run is complete, press "proceed" then "yes" when correct block is highlighted.
- 16) Carefully remove each tubes from the quad by gently lifting the top tray. Place the top tray with PCR tubes into a secondary PCR tray. Place each tube in its' appropriate location in a labeled PCR tray (forward primer, run #, date, and initials). Label the first two tubes of each 5 dilutions within a sample set
- 17) Place completed PCR tray in the freezer in lab 114. Glue the sample location template into the logbook, also in Lab 114

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Gel Preparation, Loading, Electrophoresis, and Interpretation (Lab 114)

Materials needed:


- 1) GeneMate LE Quick Dissolve® agarose
- 2) Balance
- 3) 100 mL graduated cylinder
- 4) 250 mL beaker
- 5) Microwave
- 6) Molecular Grade Reagent Water
- 7) Gloves
- 8) Safety Glasses
- 9) Working Strength (1X) Tris-borate-EDTA
- 10) GelStar® DNA stain
- 11) Orange-G®
- 12) FOTODYNE® electrophoretic chambers and power supply
- 13) FOTODYNE® UV transilluminator
- 14) Mini-gel tray with combs (12 well comb is preferred)
- 15) Appropriate molecular ladder
- 16) 0.2 mL PCR tubes
- 17) Pipettors: 0.1 – 10 µL 0.1 – 20 µL, 10 – 100 µL and assorted aerosol resistant tips. Gel loading tips are necessary for this step
- 18) Kimwipes®

Gel Preparation

When working with nucleic acid stains it is *critical* to wear gloves at all times.

- 1) A single 2% agarose gel is prepared using 40mls of 1X TBE buffer and 0.8g of agarose. After weighing out the agarose, place it into a 250 mL beaker containing the appropriate amount of 1X TBE. Preparation of 4 gels uses 3.2 grams of Agarose in 160mls of 1X TBE.
- 2) Place beaker in the microwave in the hood Lab 114. Microwave till boiling then continue to boil long enough for the agarose to fully dissolve. If preparing enough agarose for 4 gels, it will take approximately 3 minutes to thoroughly melt the agar.
- 3) Remove the beaker from the microwave but leave inside the hood to cool until it is warm to the touch but no longer hot (approximately 55°C). At this point, add the appropriate amount of GelStar® DNA stain (3-4ul per 100mls of agar).
- 4) Prepare the appropriate number of mini-gel trays by raising and locking into place the side bars and placing the comb in the appropriate slot in the chamber. Carefully pour the agarose into the

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trays; each one should contain 30-40mls of agarose. Set aside to solidify; keep shielded from light.

- 5) Electrophoretic chambers should be inspected at this point to ensure there is sufficient 1X TBE present and that it doesn't show signs of precipitation or cloudiness. Each chamber should contain approximately 200mls of 1X TBE.


Sample Preparation and Gel Inoculation

- 1) It is important to record the placement of the samples into the gel in the Laboratory logbook (e.g. sample ABC 1:10 dilution will be placed into well #1, sample XYZ 2.0 dilution will be placed in well #8, etc).
- 2) Once the gels are solidified remove the combs, lower the sides on the gel tray and place in the electrophoretic chamber with the tallest wall of the gel tray facing the electrode connections. Ensure the buffer completely covers the agarose.
- 3) Prepare and inoculate each sample individually as follows: to a tube containing 2 µL of Orange-G® add 5 µL of PCR product. Using an aerosol resistant gel tip, remove the entire 7 µL of stain and product and inoculate a well in the gel. Take care not to pierce the well with the pipet tip. Repeat this process loading all wells needed. One well of the gel should be inoculated with the molecular ladder to use as a reference point.
- 4) After all wells have been filled close tight the electrophoretic chamber. It is important to engage both the red and black electrode connection completely. Plug the red and black lines into the FOTODYNE® power supply.
- 5) Quality Control Steps:
 - a. Ensure each gel is loaded with a positive control, molecular ladder or both.
 - b. Ensure that at least one positive control and one negative control are gelled for each PCR batch.
- 6) Run the gels at 100V for approximately 1.5 hours (to ensure a minimum migration of $\frac{3}{4}$ the length of the gel). If the power supply is appropriately engaged, you will notice small bubbles forming on the positive side of the chamber. If you do not see bubbles, turn off the power supply and check electrode connections. Take care to turn off the power supply before opening the chambers.

Gel Reading and Interpretation


- 1) Turn off the power supply. Carefully open the chamber and pick up the tray taking care the gel does not slip off the tray into the chamber. Slide the gel off the tray onto the FOTODYNE® UV transilluminator, being careful not to tear the gel. Close the cover on the transilluminator, turn the power on and read the gel.
- 2) Quality Control Steps:
 - a. Ensure the ladder is well distributed

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- b. Ensure that the positive control or negative control (if present) is acceptable. If these results are questionable discard the gel and repeat the process.
- 3) Compare the molecular weight of the amplified product to the placement on the ladder. For *Bacteroides spp*, the weight should be as follows: 700 base pair (bp) for the general *Bacteroides* and between 515 and 580 bp for the host specific assays.
- 4) If the bands are easily visible and match up in the proper weight area, record as a positive result in the Laboratory logbook for that sample number/well number. Should bands be absent or in the wrong location, record as a negative result in the logbook. Do this for each well raceway and for each gel.
- 5) Discard the gel after results have been recorded.

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Attachment 1.

Master Mix Volumes (in uls)


No. of samples to amplify --->	1	5	8	10
DNase-free water	94.5	472.5	756	945
10X Ex Taq buffer	13.5	67.5	108	135
dNTP	10.8	54	86.4	108
25% Acetamide Solution	5.4	27	43.2	54
Forward Primer	2.7	13.5	21.6	27
Reverse Primer (708R)	2.7	13.5	21.6	27
Ex Taq Enzyme (add last)	0.675	3.375	5.4	6.75

Attachment 2.

96-well template

		1	2	3	4	5	6	7	8	9	10	11	12
Primer/date/initials	A												
	B												
	C												
	D												
	E												
	F												
	G												
	H												

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Document History

Document history					
Version #	Status (I, R, C)	Date Approved	Location of Change History	Name & Title	
				Author	Approving Official
1	I	8/24/2015	N/A	Stephanie Bailey, Microbiologist	Reviewer: Dana Walker, Quality Assurance Coordinator Approver: Dana Walker, Quality Assurance Coordinator
					Reviewer: Approver:
					Reviewer: Approver:

Version #: version number of this document.

Status: I = Initial, R= Revision, or C = Cancelled.

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Appendix C:

Microbiology Laboratory Data Review/Release Form

Project:

Project Code:

Sample Numbers Peer Reviewed by:

Date:

Raw Data/Quality Control Check

- _____ Verify positive and negative culture controls associated with media are satisfactory.
- _____ Verify media sterility was checked.
- _____ Check for sample carryover/contamination if membrane filtration method used. Note any deficiencies.
- _____ Check duplicate analyst counts are within 20 %, when applicable.
- _____ Verify that media was prepared within method specifications.
- _____ Verify that samples were received and analyzed within the holding time.

Bench Sheet Check

- _____ Is the data package properly labeled?
 - _____ Analyst name
 - _____ Sample numbers and project name
 - _____ Analytical method used
 - _____ Date and time of collection/analysis
- _____ Verify that there is a bench sheet for each sample listed on the Analysis Required forms.
- _____ Verify that there is a Data Review Memo written for the project -forwarded to ESAT Data Entry Technician
- _____ Verify that there is a Data Release Memo for this project - forwarded to ESAT Data Entry Technician

Results

- _____ Verify that the reported results:
 - _____ have appropriate qualifiers assigned
 - _____ reflect the correct units
 - _____ reflect dilution factors used in the analysis
 - _____ were transferred correctly from the bench sheets
 - _____ were calculated correctly

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Appendix D:

MST Sample Collection Schedule

The table below provides a summary example of the Nooksack River Watershed MST Study sample schedule. One duplicate sample will be collected per sampling event, with the site selected for duplicate sampling selected by the study partners in advance. Duplicate sampling will be targeted to provide duplicate sampling during the rainy season for all sites, and priority duplication for sites for which additional information would be most useful.

Table 13.1 Summary Sampling Schedule for Nooksack River Watershed MST Study

Sampling Event	Duplicate	Sterile Transfer
January 2016	Portage Bay (DH050)	Whatcom County
February 2016	Lower Nooksack River (SW118)	LWRD
March 2016	Upper Nooksack River (M5)	Whatcom County
April 2016	Fishtrap Creek (F1)	LWRD
May 2016	Bertrand Creek (B1)	Whatcom County
June 2016	Kamm Creek (K1)	LWRD
July 2016	Portage Bay (DH050)	Whatcom County
August 2016	Lower Nooksack River (SW118)	LWRD
September 2016	Upper Nooksack River (M5)	Whatcom County
October 2016	Scott Ditch (S1)	LWRD
November 2016	Tenmile Creek (T1)	Whatcom County
December 2016	Fishtrap Creek (F1)	LWRD

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Appendix E:

Data Quality Objectives Summary – Laboratory Measurements

Table 13.2 Data Quality Objectives Summary

Analytical Group	Number of Samples per sampling event ²	Total Number of Samples for Project ³	# of QA Samples: Reference Samples	Matrix	Method	Method Detection Limits	Accuracy	Precision (RPD)	Completeness	Volume, Container	Holding Time (days)
Filtration	10	120	1 each batch	Water	ORD prep by filtration	1 strand DNA	See Section 4.3	See Section 4.3	> 90 %	250 ml PP, sterile	30 hours
PCR	Varying	Varying	1 each batch	Frozen prepared filter	<i>Bacteriodes</i> identification	1 strand DNA	See Section 4.3 ¹	See Section 4.3 ¹	> 90 %	N/A	None (after filtration)

¹ – Standard Accuracy and Precision for analysis by PCR is unknown at this time. Identification is not quantitative.

² – Number of samples includes 8 field samples, a field duplicate, and a field blank (10 total)

³ – Total Number of samples = 120: 10 samples per event (including field QC) for 12 events. Varying number of PCR samples depends on the number of primers required.

PP: Polypropylene